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Contribution to the knowledge of the metabolism of Listeria
monocytogenes (type 1) with special consideration of oxidation of
carbohydrates and metabolites of the tricarbonic acid cycle and its
response to inhibitors.

by E. Kolb and H. Seidel.

I. Introduction.

The last few years have seen the publication of numerous clinical,
bacteriological, serological and morbid anatomical papers on problems
of listeriosis, as evident from Seeliger's monograph (1955) and the
symposium on listeriosis by Roots and Strauch (1958), to mention just
a few. Relatively few studies deal with the nutrient requirements of
Listeria monocytogenes or its fermentive equipment and metabolic
properties. The clarification of these questions is indicated
principally from the biochemical point of view, but touches also on
practical problems of chemotherapy and cultivation on synthetic media.
The present paper describes the response of the resting respiration of
Listeria monocytogenes to different natural substrates under variable
conditions of oxidation, and the effect on respiration of various
antibiotics and ferment inhibitors.

II. Literature.

Listeria monocytogenes generally takes a coccoid form and measures
0.5 - 0.8/0.6 - 1.2 microns, although filamentous forms have been
described (Bergey, Seeliger and Linzenmeier, Schulz, Claasen). The
bacterium is grampositive with a tendency to gramlability. It shows
lively motility at $+22^{\circ}C$, made possible by peritrichous flagellation.

A number of studies deals with the question of the influence of
temperature on growth. According to Hein as well as Seeliger and
Linzenmeier, slow growth is still perceptible at $+4^{\circ}C$. Bergey,
Seeliger, Schulz as well as Seeliger list $+37^{\circ}C$ as the optimal temper-
ature. The upper growth limit is near $+42$ to $44^{\circ}C$, according to
Seeliger. The thermal death point, according to Bergey or Seeliger, is
near $+58$ to $59^{\circ}C$ when held for 10 minutes, or near $+80^{\circ}C$ for 15 seconds.

Listeria monocytogenes is a facultative aerobe with microaerophilic or carbodioxidophilic properties (Hein, Seeliger). Quantitative studies of the oxygen consumption of resting and growing bacterial cultures have not been found in the available literature.

Roots has presented electron-microscopic studies of the cell wall structure of Listeria monocytogenes. The bacterium's cell walls are relatively susceptible to mechanical influences. Listeriae have proved to be quite sensitive to proteolytic ferments. The presence of relatively strong bacterial membranes must be considered in evaluating the results of bacterial metabolic studies, since these frequently represent a limiting factor in absorption or during transport of the substrate to the degrading ferment system. Thus numerous findings in the literature indicate that natural substrates utilized by the bacterium for syntheses and energizing reactions do not effect respiratory stimulation due to their relatively slow passage through the cell membranes.

Noteworthy biochemical properties are: A positive catalase reaction, negative indole, no production of hydrogen sulfide, no reduction of nitrate and no gelatine liquefaction. Growth is possible within the range of pH 5.6 - 9.0. The following compounds are fermented: Glucose, fructose, rhamnose, salicin, aesculin and trehalose; slight fermentation is demonstrable in the presence of maltose and dextrin. On the other hand, arabinose, dulcitol, inositol, adonidin, inulin and mannitol are not degraded with acid production. Data on the fermentation of galactose, lactose, saccharose, sorbitol, glycerol, xylose and starch are inconsistent (Bergey, Seeliger and Linzenmeier, Hein, Schulz as well as Roots and Strauch).

The factor of Listeria monocytogenes that produces monocytosis in different animal species is a lipoid extractable with chloroform from mechanically disintegrated cells. No studies of the enzymatic equipment of Listeria monocytogenes are known to date, which is true also of its nutritional requirements; the organism is generally considered to have modest needs.

III. Personal investigations.

A. Methods.

The present study used a Listeria strain of type 1 isolated by Seeliger. The organism was grown in nutrient broth (pH 7.2) over 3 to 4 days at 37°C under aerobic conditions. Next, the bacterial cultures were centrifuged for 15 minutes at 5,000 RPM; the resultant sediments were purified twice with m/10 phosphate buffer (pH 7.2). The listerial suspension, freed of most nutrients by this washing process, was taken up with m/10 phosphate buffer at pH 7.2 and shaken with glass beads to obtain better diffusion. Cultures that were not used immediately were stored at /4°C. Manometric studies of respiratory processes were

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carried out in the Warburg apparatus according to the method described in detail by Bertho and Grassmann, Bamann and Myrbaeck, as well as Umbreit, Burris and Stauffer.

The metabolites tested in the present study were added to the resting bacterial suspension in known concentrations. 1.5 ml bacterial suspension and 0.5 ml substrate solution were used for this purpose. Blank respiration was determined by initiation 1.5 ml bacterial suspension with 0.5 ml phosphate buffer, usually followed by pre-incubation for one hour. Absorption of CO_2 liberated during metabolic action was made with 20% potash lye.

All tests were adjusted to a uniform manometer shaking frequency of 80 beats. Utilization of oxygen was determined hourly over a period of 5 hours. Dual determinations were made in every case; test results were rechecked in repeated batches.

The absence of a uniform basis of oxygen consumption frequently impedes the comparison of metabolism in different microorganisms. Lately, analysis os oxygen absorption per mg dry substance is being replaced by reference to mg nitrogen. In the present tests, two dual analyses of the bacterial suspension's nitrogen content were made for each culture according to Kjeldahl's method, and oxygen consumption was computed uniformly per 0.05 mg nitrogen.

B. Test results.

1. Oxidation tests.

The results of blank respiration of Listeria monocytogenes at different temperatures as well as its response to addition of various substrates are represented below (Table 1 and 2). In every case, blank respiration and respiration upon addition of the substrate were analysed at hourly intervals. The progress of oxygen utilization of a substrate with strong respiratory stimulation (glucose) is shown in Fig. 1, that of a compound with low inducement of oxidation (arabinose) in Fig. 2.

Fig. 1 and 2 reveal that glucose increases the blank respiration of Listeria monocytogenes $4\frac{1}{2}$ times at $/37°C$, whereas arabinose is far less effective. Considerable differences in effect on blank respiration were demonstrated also among other substrates that may be used by microorganisms in general. In view of the variable motility of listeriae at $/22°C$ and at $37°C$, it seemed fruitful to examine the action of basal respiration at different temperatures with and without addition of substrates. The tests showed that the blank respiration of Listeria monocytogenes increases considerably with rising temperatures. While its range of O_2 absorption was 19-35 microliters upon incubation of cultures at $/22°C$, it rose appreciably during increase in temperature to $/37°C$ and covered a range of 63-91 microliters O_2 consumption per

0.05 mg nitrogen over a period of 5 hours. Even higher values were seen at $/45^{\circ}$C, where blank respiration yielded values of 105-118 microliters O_2.

Values listed in Table 1 represent pure respiratory stimulation obtained by subtraction of blank respiration (without addition of substrate) from respiration with substrate. It is evident that stimulation due to different substrates is extraordinarily variable. Pantothenic acid, mannitol and sorbitol have no effect or a very slight effect. Of the disaccharides, lactose is foremost in its stimulating effect on the respiration of Listeria monocytogenes. The effect of glucose is considerably stronger than that of fructose. Strongly stimulating substrates generally have their optimum at $/37^{\circ}$C; lactose and arabinose promoted oxidation more at $/45^{\circ}$C than at $/37^{\circ}$C. In agreement with metabolic conditions of resting respiration, absorption of oxygen was relatively low at $/22^{\circ}$C even in the presence of substrates with strong powers of stimulation.

Table 2 lists both blank respiration and additional oxygen absorption in the presence of 3 metabolites of the tricarbonic acid cycle. It is evident that sodium succinate as well as sodium malate are very easily oxidized by the bacterial cell. Sodium-alpha-ketoglutarate, on the other hand, has only a slight effect under the given conditions. It must be noted in this connection that no additional stimulation due to succinate and malate is evident from $/37^{\circ}$C to $/45^{\circ}$C when compared to considerably increased blank respiration. This indicates that increased oxidation at higher temperatures is supported primarily by realease of endogenic reserves.

Studies of different bacteria have revealed that partial or absent respiratory stimulation due to metabolites of the tricarbonic acid cycle may be blamed on the poor passage of these compounds through the cell membrane (Werkman and Wilson). This limiting factor usually can be removed by prolonged incubation of the bacterial suspension with the substrate.

Additional tests were designed to determine the effect of permeability on oxidation of succinate, malate and alpha-ketoglutarate with extended pre-incubation times at $/37^{\circ}$C. It was established that no appreciable effect is evident upon prolonged pre-incubation with glucose. Studies with malate, succinate and alpha-ketoglutarate, on the other hand, revealed considerable promotion of oxidation upon prolonged pre-incubation, as reflected in Table 3.

Other tests were devoted to the study of the effect of substrate concentration on oxidation. As already stated, previous tests utilized a terminal concentration of m/40. In subsequent investigations, 0.5 ml of an m/50, m/100 or m/200 solution were added, resulting in a terminal concentration of m/200, m/400 or m/800.

Table 4 shows that the stimulating effect of the substrate diminishes rapidly with decreasing substrate concentrations until, at end concentrations of m/400 or m/800, no influence on blank respiration is noted.

2. Inhibition of oxidation.

Additional tests investigated the effect of different chemotherapeutics and antibiotics such as salthion, penicillin, streptomycin and hostacyclin as well as of a few ferment inhibitors (KCN, NaF) on the respiration of Listeria monocytogenes. Bacterial suspensions were used in every case, to which were added 0.5 ml m/10 glucose solution and inhibitors in concentrations indicated.

Fig. 3 shows the hourly progress of glucose oxidation and its response to hostacyclin in concentrations of 1 and 100 γ per batch.

While addition of hostacyclin in a terminal concentration of 100 γ per batch (5 mg%) induces considerable inhibition of respiration (more than 50%), the weak concentration proves to be far less effective. Further results of inhibition tests are listed in Table 5.

The strongest effect on glucose oxidation is produced by penicillin and hostacyclin, both of which inhibit by more than 50% at highest concentrations. According to Seeliger, the tetracyclins are most promising for treatment of listeriosis with antibiotics, as evidenced by inhibition tests on nutrient media. It is noteworthy in this connection that the resting respiration of bacteria is generally less susceptible to antibiotics than the metabolism of growing bacteria, as revealed in studies of this phenomenon by Goetz and others. The causes are not clear in detail. The dissimilar permeability of the cell membrane probably is an important factor, since greater permeability and a more intensive transport of substances into the cell must be assumed during growth, at the same time admitting inhibitors on a larger scale.

Addition of potassium cyanide and sodium fluoride induced only a slight inhibition of resting respiration under glucose enrichment. At a terminal concentration of m/40, KCN produced 7% inhibition, NaF, 16%.

IV. Discussion of results.

The carbohydrates fermented by Listeria monocytogenes within 24 hours (glucose, fructose and maltose were tested) were foremost with respect to their stimulation of respiration, according to studies by Seeliger and Linzenmeier. Carbohydrates that are not fermented or used only to a small extent (saccharose, sorbitol, mannitol and arabinose) were considerably less effective or without appreciable influence. An exception was noted in the case of lactose, which does not produce acid but induced a strong respiratory stimulation in the tests under discussion.

Total respiration of Listeria monocytogenes in the presence of glucose in a terminal concentration of m/40 and a temperature of $+37^\circ C$ was around 300 microliters O_2 per 0.05 mg nitrogen.

In order to offer comparisons between the respiratory intensity of Listeria monocytogenes and other bacterial species, a few values have been given below (Table 6), in which oxygen consumption is related to mg dry weight per hour.

Since our tests did not determine dry weight, the respiratory intensity of Listeria monocytogenes cannot be compared directly with the organisms listed in Table 6. When the fact is considered that the dry fraction of bacteria generally amounts to 20% and the protein fraction approximately 15%, the total respiration of Listeria monocytogenes yields an oxygen absorption rate of about 60 microliters per hour and per 0.4 mg dry substance; this is about 150 microliters per mg dry substance. According to this computation, the respiratory intensity of Listeria monocytogenes is fairly high and approaches the range of very active organisms, e.g. Escherichia coli.

As already established in connection with other bacteria, the respiratory intensity rises considerably with increasing temperatures. The present study has pointed out that a very intensive resting respiration is still demonstrable at $+45^\circ C$, although the upper growth limit of Listeria monocytogenes has been listed at $+42$ to $44^\circ C$.

Respiratory stimulation due to sodium malate and sodium succinate is remarkably high, as is that of sodium alpha-ketoglutarate after prolonged incubation. This allows the conclusion that Listeria monocytogenes utilizes the ferment chain of the tricarbonic acid cycle which, among others, effects the final degradation of carbon chains and which has been demonstrated previously in numerous bacteria. The relatively weak respiratory inhibition upon addition of glucose and sodium fluoride indicates that glucose is not decomposed to any appreciable degree via the ferment chain of glycolysis. Potassium cyanide inhibits respiration even less. As in many other bacteria, a non-ferrous respiration consequently is paramount in Listeria monocytogenes.
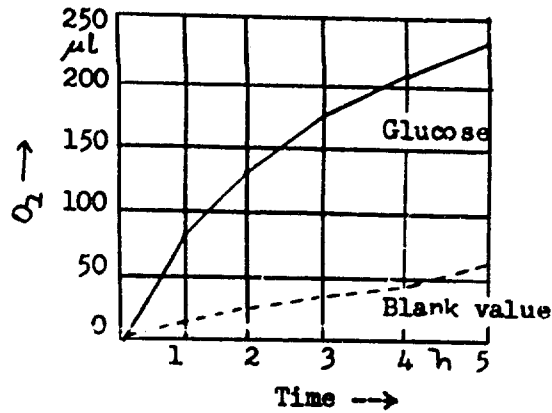
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Fig. 1. Oxygen consumption of Listeria monocytogenes with and without addition of glucose (end concentration m/40) at ⨍37°C and preincubation for 1 hour at ⨍37°C.
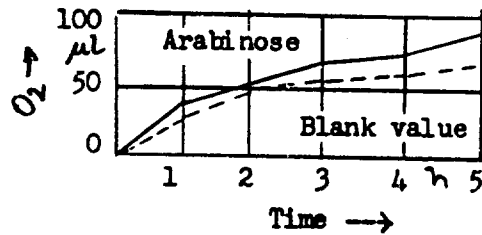

Fig. 2. Oxygen consumption of Listeria monocytogenes with and without addition of arabinose (end concentration m/40) at ⨍37°C and preincubation for 1 hour at ⨍37°C.
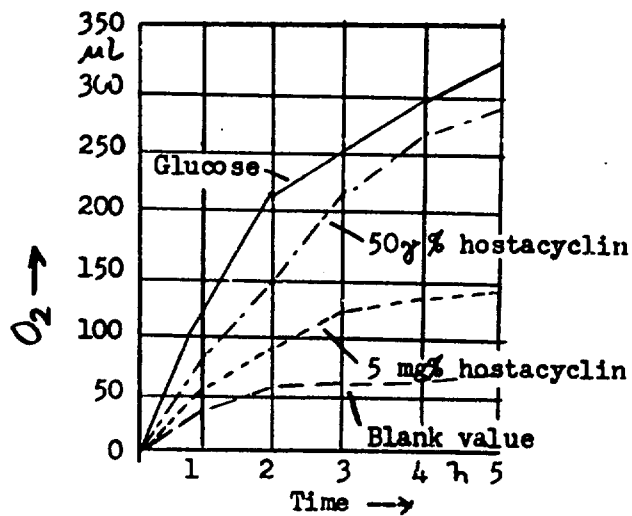

Fig. 3. Response of oxygen consumption of Listeria monocytogenes to hostacyclin at ⨍37°C in the presence of glucose.

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Tables

Table 1. Stimulation of basal respiration of Listeria monocytogenes by different substrates at various temperatures in microliters O_2 per 0.05 mg N in 5 hours.

Added substrate	Temperature		
	$/22°$	$/37°$	$/45°$
Glucose	64	232	218
Fructose	34	134	104
Maltose	17	100	98
Lactose	19	107	220
Saccharose	8	41	29
(/) Arabinose	2	18	19
D-mannitol	3	5	5
D-sorbitol	-	2	2
Pantothenic acid	-	-	-

Table 2. Stimulation of basal respiration of Listeria monocytogenes by different substrates of the tricarbonic acid cycle in microliters O_2 per 0.05 mg N in 5 hours.

Added substrate	Temperature		
	$/22°$	$/37°$	$/45°$
Sodium malate	15	140	141
Blank respiration	20	68	110
Sodium succinate	16	150	143
Blank respiration	29	65	112
Sodium alpha-ketoglutarate	4	18	17
Blank respiration	30	78	115

Table 3. Stimulation of basal respiration of Listeria monocytogenes by malate, succinate and alpha-ketoglutarate in microliters O_2 per 0.05 mg N in 5 hours with pre-incubation for 1 and 20 hours at $/37°C$.

Added substrate	Pre-incubation	
	1 hour	20 hours
Sodium malate	140	275
Sodium succinate	150	231
Sodium alpha-ketoglutarate	18	167
Glucose	232	220

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Table 4. Effect of substrate concentration on the respiration of Listeria monocytogenes at ⧸37°C.

Added substrate	Substrate end concentration			
	m/40	m/200	m/400	m/800
Glucose	232	66	-	-
Sodium malate	140	18	-	-
Sodium succinate	150	20	-	-
Sodium alpha-ketoglutarate	18	18	-	-

Table 5. Response of glucose oxidation of Listeria monocytogenes to different inhibitors.

Inhibitor	End concentration	Inhibition in %
Salthion	0.25 mg%	0
	2.50 mg%	23
	25.00 mg%	43
Penicillin	1 unit/2 ml	30
	10 units/2 ml	46
	100 units/2 ml	56
Streptomycin sulfate	50 γ %	9
	500 γ %	13
	5.00 mg%	37
Hostacyclin	50 γ %	9
	5.00 mg%	55

Table 6. Oxygen consumption of some bacteria in microliters O_2 per mg dry weight per hour in the presence of glucose (after Albritton).

Bacterial species	Temperature in ⧸°C	Oxygen utilization
Escherichia coli	40	200
Micrococcus luteus	35	15
Pseudomonas fluorescens	26	58
Streptococcus faecalis	38	106
Streptococcus pyogenes	37.5	25 - 42
Bacillus subtilis	37	170
Lactobacillus bulgaricus	37	34